Pflugers Arch – Eur J Physiol (2005) 449: 392–402
DOI 10.1007/s00424-004-1351-9

EPITHELIAL TRANSPORT

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Expression and regulation of the renal Na/phosphate cotransporter NaPi-IIa in a mouse model deficient for the PDZ protein PDZK1

Received: 23 July 2004 / Accepted: 16 September 2004 / Published online: 29 October 2004

Abstract Inorganic phosphate (P_i) is reabsorbed in the renal proximal tubule mainly via the type-IIa sodium-phosphate cotransporter (NaPi-IIa). This protein is regulated tightly by different factors, among them dietary P_i intake and parathyroid hormone (PTH). A number of PDZ-domain-containing proteins have been shown to interact with NaPi-IIa in vitro, such as Na^+/H^+ exchanger-3 regulatory factor-1 (NHERF1) and PDZK1. PDZK1 is highly abundant in kidney and co-localizes with NaPi-IIa in the brush border membrane of proximal tubules. Recently, a knock-out mouse model for PDZK1 ($Pdzk1^{-/-}$) has been generated, allowing the role of PDZK1 in the expression and regulation of the NaPi-IIa cotransporter to be examined in in vivo and in ex vivo preparations. The localization of NaPi-IIa and other proteins interacting with PDZK1 in vitro [Na^+/H^+ exchanger (NHE3), chloride-formate exchanger (CFEX)/putative anion transporter-1 (PAT1), NHERF1] was not altered in $Pdzk1^{-/-}$ mice. The abundance of NaPi-IIa adapted to acute and chronic changes in dietary P_i intake, but steady-state levels of NaPi-IIa were reduced in $Pdzk1^{-/-}$ under a P_i rich diet. This was paralleled by a higher urinary fractional P_i excretion. The abundance of the anion exchanger CFEX/PAT1 (SLC26A6) was also reduced. In contrast, NHERF1 abundance increased in the brush border membrane of $Pdzk1^{-/-}$ mice fed a high-P_i diet. Acute regulation of NaPi-IIa by PTH in vivo and by PTH and activators of protein kinases A, C and G (PKA, PKC and PKG) in vitro (kidney slice preparation) was not altered in $Pdzk1^{-/-}$ mice. In conclusion, loss of PDZK1 did not result in major changes in proximal tubule function or NaPi-IIa regulation. However, under a P_i-rich diet, loss of PDZK1 reduced NaPi-IIa abundance indicating that PDZK1 may play a role in the trafficking or stability of NaPi-IIa under these conditions.

Keywords PDZ proteins · Proximal tubule · Phosphate transport · Brush border membrane · NHERF · PDZK1 · Mouse

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Introduction

Inorganic phosphate (P_i) reabsorption in renal proximal tubules is mediated mainly by the type-IIa sodium-phosphate cotransporter (NaPi-IIa) [20, 21, 23]. This protein is regulated tightly by different factors such as dietary P_i intake, systemic acid-base status and hormones [20]. Among these parathyroid hormone (PTH) plays an important role, increasing the rate of endocytosis of the cotransporter via activation of a complex signalling network involving different protein kinases such as PKA and PKC [22].

In a recent yeast two-hybrid screen we have identified several proteins interacting with the C-terminal TRL motif of NaPi-IIa [8]. These proteins include Na^+/H^+ exchanger-3 regulating factor-1 (NHERF1) and PDZK1 (formerly mouse NaPi-Cap1) that contain multiple PDZ domains. The interaction between NaPi-IIa and PDZK1 and NHERF1 requires the PDZ-binding motif of NaPi-IIa and maps to the PDZ domain 1 of NHERF1 and PDZ domain 3 of PDZK1 [8, 10]. In addition, the interactions have been confirmed further in in vitro assays such as overlays, pull-downs and co-immuno-precipitations from native tissue as well as from cells [8, 9, 10, 26].

The role of NHERF1 as an important protein regulating NaPi-IIa function in the proximal tubule has emerged from several lines of evidence. Disruption of the interaction between the C-terminal TRL motif of NaPi-IIa and PDZ domain 1 in opossum kidney (OK) cells results in loss of apical positioning of NaPi-IIa [11]. Similarly, apical expression of NaPi-IIa in the brush border membrane is reduced in NHERF1-deficient mice [25]. In addition, loss of NHERF1 seems to disrupt the normal adaptive up-regulation of NaPi-IIa with a low dietary P_i intake or after switching the culture medium to a low P_i content [5, 28]. In addition, PTH-induced down-regulation of NaPi-IIa is altered in primary cell culture [5] or in freshly isolated kidney slices derived from NHERF1-deficient mice [27]. Thus, NHERF1 appears to play a role in positioning and regulating NaPi-IIa.

PDZK1 was originally identified as interacting with the membrane-associated protein of 17 kDa (MAP17) in a yeast two-hybrid screen [14]. The same method also showed interaction of PDZK1 with NaPi-IIa and, via its four PDZ domains, also with other proteins expressed in the proximal tubule such as NHE3, chloride-formate exchanger/putative anion transporter-1 (CFEX/PAT1; SLC26A6), the organic cation transporter OCTN1, the urate transporter URAT1, NHERF1, the protein kinase A anchoring protein D-AKAP2, and MAP17, a protein of unknown function [9, 10, 24]. The role of PDZK1 in the proximal tubule has remained unclear due to the lack of appropriate cell culture models. However, it has been suggested that PDZK1 could form, together with NHERF1 and other scaffolding proteins, a network of proteins involved in the trafficking, apical positioning and regulation of various transporters, receptors, other components of signalling cascades and proteins mediating endo- or exocytosis of these complexes [9, 10, 12].

The recent generation of a PDZK1-deficient mouse model has allowed us to study the role of PDZK1 in the expression, localization and regulation of the NaPi-IIa cotransporter in in vivo and in ex vivo preparations [15]. This study showed that loss of PDZK1 did not result in major changes in proximal tubule function and NaPi-IIa regulation. However, under a P_i-rich diet, loss of PDZK1 reduced NaPi-IIa abundance and increased fractional P_i excretion, indicating that PDZK1 may play a role in the trafficking or stability of NaPi-IIa under these conditions.

Materials and methods

Animal studies

Experiments were performed with age- and sex-matched wild-type mice 129SV/EV (*Pdzk1*$^{+/+}$) and *Pdzk1* knock-out mice (*Pdzk1*$^{-/-}$) with the same genetic background, weighing 30–35 g at 22–24 weeks of age. The generation, breeding, and genotyping of these mice has been described previously [15, 16]. Animals were housed in climatized animal facilities and received diets (Kliba, NAFAG, Switzerland) with a high (1.2%) or low (0.1%) P_i content and had free access to water. For some experiments mice were trained to receive food for only 1 h daily to time food intake.

Spontaneous urine samples were collected daily at the same time and frozen rapidly until further analysis. Blood samples were collected immediately before sacrificing the mice by puncture of the vena cava. All samples were analysed for P_i and creatinine using commercial kits (Sigma Diagnostics, St. Louis, Mo., USA and Wako Chemicals, Neuss, Germany) according to the manufacturers' protocols. All animal studies were approved by Harvard Medical School Animal Care Committee.

Western blot analysis

Mice were anaesthetized with ketamine-xylazine i.p., perfused through the left ventricle with warm (37 °C) sucrose/phosphate buffer (140 mM sucrose, 140 mM NaH_2PO_4/NaH_2PO_4, pH 7.4) and the kidneys removed rapidly and frozen until further analysis. Frozen kidneys were used for brush border membranes preparation as described previously using the Mg^{2+}-precipitation technique [4].

For Western blots, brush border membrane protein concentration was measured (Biorad Protein kit) and 10 μg protein solubilized in Laemmli sample buffer containing 2% (v/v) 2-mercaptoethanol. SDS-PAGE was performed on 10% polyacrylamide gels. Proteins were transferred electrophoretically from gels to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, Mass., USA). After blocking with 5% milk powder in TRIS-buffered saline containing 0.1% Tween-20 for 60 min, the blots were incubated with the primary antibodies (rabbit anti-PDZK1 [8] 1:500, rabbit anti-NaPi-IIa 1:6,000 [6], mouse monoclonal anti-actin (Sigma), rabbit anti-NHERF1/2 (kindly provided by E. Weinman, University of Maryland) 1:4,000, rabbit anti-CFEX (kindly provided by P.S. Aronson, Yale University [13]) 1:1,000, rabbit anti-MAP17 [24] 1:4,000), rabbit anti-NHE3 (kindly provided by O.W. Moe, University of Texas, Dallas) 1:5,000 overnight at 4 °C or 2 h at room temperature. After washing and subsequent blocking, blots were incubated with the secondary antibodies (donkey anti-rabbit 1:10,000 or sheep anti-mouse 1:10,000, respectively) IgG-conjugated with horseradish peroxidase (Amersham Life Sciences) or IgG-conjugated with alkaline phosphatase (Promega, Madison, Wisc., USA) for 1 h at room temperature. Antibody binding was detected with the peroxidase/luminal enhancer kit (Pierce, Rockford, Ill., USA) or with CDP-Star (Roche Diagnostics, Indianapolis, Ind., USA) respectively, using the DIANA III-chemiluminescence detection system (Raytest, Straubenhardt, Germany). All images were analysed using appropriate

software [Advanced Image Data Analyser (AIDA), Raytest] to calculate the protein of interest/actin ratio. The significance of differences between means was determined using Student's *t*-test for unpaired samples. $P < 0.05$ was considered significant.

PTH injection

For PTH injection, four *Pdzk1*$^{+/+}$ and four *Pdzk1*$^{-/-}$ mice were fed a low-P_i diet for 4 days to increase the expression of the NaPi-IIa protein in the kidney. Mice were then injected i.p. with 0.5 μg/g BW 1–34 fragment of PTH (Sigma) or with 0.9% NaCl as control and sacrificed after 90 min. Kidneys were harvested rapidly and frozen until further use. Urine samples were collected before the injection and immediately before sacrifice. Blood samples were collected immediately before sacrifice. All urine and serum samples were frozen rapidly until further use.

Kidney slices

Kidney slice experiments were performed as described previously [2, 3]. Briefly, mice were kept on a low-P_i diet for 5 days prior to the experiments to increase NaPi-IIa expression in the kidneys. Mice were anaesthetized and perfused through the left ventricle with 50 ml warm (37 °C) sucrose/phosphate buffer to remove all blood from the kidneys. Kidneys were harvested rapidly, adhering connective tissue and extrarenal vessels removed and thin coronal slices (about 1 mm thick) cut. From each kidney six or seven slices could be prepared. Slices were transferred into 4 ml pre-warmed (37 °C) Hank's buffer (in mM: NaCl 110, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 1.8, Na-acetate 4, Na-citrate 1, glucose 6, L-alanine 6, NaH_2PO_4 1, Na_2HPO_4 3, $NaHCO_3$ 25, pH 7.4, gassed with 5% CO_2 and 95% O_2) and allowed to adapt for 10 min at 37 °C in a water bath before the start of the incubation. Slices were then left untreated (control) or incubated with 1–34-PTH (100 nM), 3–34-PTH (100 nM), 8-Br-cAMP (100 μM), 8-Br-cGMP (1 mM) or 1,2-dioctanoyl-sn-glycerol (DOG, 10 μM). During the whole course of the experiments all solutions were gassed with 5% CO_2/95% O_2 and the pH was kept constant at pH 7.4 ± 0.1.

Chemicals were from Sigma unless stated otherwise. All experiments were performed at least with two kidneys from two different animals. Untreated slices were used in all experiments as an internal control. For immunohistochemistry, kidney slices were transferred to a fixation solution (3% paraformaldehyde) at the end of the incubation [3] and fixed for 4 h on ice. After fixation, slices were rinsed few times with PBS, mounted on thin cork plates and frozen immediately in liquid propane cooled in liquid nitrogen.

Immunohistochemistry

Mice were fixed by perfusion through the left ventricle as described previously [7]. After perfusion, the kidneys were removed and cut into slices that were mounted on thin cork plates and frozen immediately in liquid propane cooled with liquid nitrogen. Cryosections (4 μm thick) mounted on chrome alum/gelatine-coated glass slides were processed for immunofluorescence. For NaPi-IIa, NHE3 and MAP17 immunofluorescence stains, sections were pretreated for 10 min with 3% defatted milk powder and 0.02% Triton X-100 (blocking solution) in PBS. After rinsing with PBS, sections were incubated with rabbit anti-rat NHE3 diluted 1:1,000, or with rabbit anti-rat serum against the NaPi-IIa protein diluted 1:500 or with rabbit anti-rat MAP17 diluted 1:1,000. For immunofluorescence detection of PDZK1 and D-AKAP2 sections were microwaved in a buffer containing 0.01 M citrate in distilled water at 30% power for 10 min. After rinsing with PBS and covering for 10 min with blocking solution, sections were incubated over night with anti PDZK1 antibody diluted 1:500 or with anti D-AKAP2 diluted 1:1,000. For antibodies against NHERF1/2, CFEX and the Na-sulphate transporter-1 (NaSi-1) sections were pretreated with 0.5% SDS in PBS for 7 min. After repeated rinsing with PBS they were covered for 10 min with blocking solution. Sections were then incubated overnight at 4 °C with anti NHERF antibody diluted 1:500, or with anti CFEX diluted 1:30 [13] or with rabbit anti-rat serum against the NaSi-1 protein [18] diluted 1:500. All primary antibodies were diluted in blocking solution and incubated overnight at 4 °C. Sections where then rinsed 3 times with PBS and covered for 45 min at room temperature in the dark with secondary antibody coupled to fluorescein isothiocyanate (FITC) or indocarbocyanine (CY3) (Dakopatts, Glostrup, Denmark). Double staining for NaPi-IIa and β-actin filaments was achieved by adding rhodamine-phalloidin (Molecular Probes, Eugene, Ore., USA, 1:50) in the solution containing the secondary antibodies. After rinsing with PBS, the sections were finally covered with glass-slips by using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo (2.2.2.)octane (DABCO, Sigma) as a fading retardant, and inspected using epifluorescence microscopy (Polyvar, Reichert-Jung).

Results

Steady-state expression of the NaPi-IIa cotransporter

Renal P_i reabsorption, localization and abundance of the NaPi-IIa protein are regulated tightly by dietary P_i intake. A diet rich in P_i reduces NaPi-IIa abundance, whereas a low-P_i diet increases NaPi-IIa expression [17]. We therefore first measured urinary P_i excretion and the

abundance and localization of NaPi-IIa in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice kept for 5 days (steady-state) on low- or high-P_i diets.

Pdzk1$^{+/+}$ and *Pdzk1*$^{-/-}$ mice fed a diet with a low (0.1%) inorganic P_i content for 5 days had a low urinary P_i excretion (expressed as mg P_i/mg creatinine), which was similar in both groups (Fig. 1a). The abundance of NaPi-IIa in the brush border membrane and its localization did not differ in kidneys from wild-type and PDZK1-deficient mice. Strong staining of the brush border membrane of early proximal tubules both of superficial and juxtamedullary nephrons was observed (Fig. 1b,c and Fig. 3).

A second group of mice received a diet with high (1.2%) inorganic P_i content for 5 days. In this group, total and fractional urinary P_i excretion was significantly higher in *Pdzk1*$^{-/-}$ mice than in wild-type animals (Fig. 2a, Table 1). Similarly, NaPi-IIa protein abundance in the brush border membrane was significantly lower in the *Pdzk1*$^{-/-}$ than in *Pdzk1*$^{+/+}$ mice

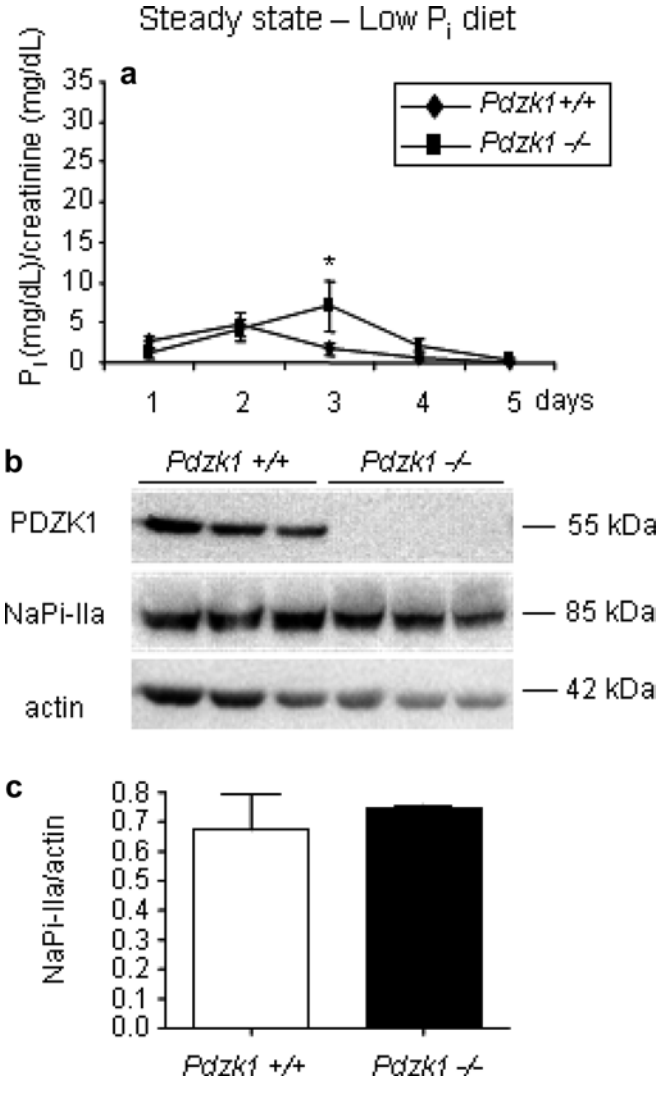

Fig. 1a–c Urinary P_i excretion and NaPi-IIa abundance in wild-type (*Pdzk1*$^{+/+}$) and PDZK1-deficient (*Pdzk1*$^{-/-}$) mice fed a low-P_i diet (0.1% P_i content) for 5 days. **a** Urinary P_i excretion relative to creatinine excretion (mg Pi/mg creatinine) measured in spontaneous urine samples collected daily at the same time from both genotypes. *Points* represent the average of the values obtained from three to five animals. *$P < 0.05$. **b**, **c** Western blot analysis for the Na/phosphate cotransporter (*NaPi-IIa*) in renal brush border membranes from wild-type and PDZK1 knock-out mice after 5 days on the low-P_i diet. *Upper panel*: PDZK1 protein expression; *middle panel*: NaPi-IIa expression. All membranes were stripped and reprobed for actin (*lower panel*) to normalize for loading. **c** Abundance of NaPi-IIa relative to that of actin

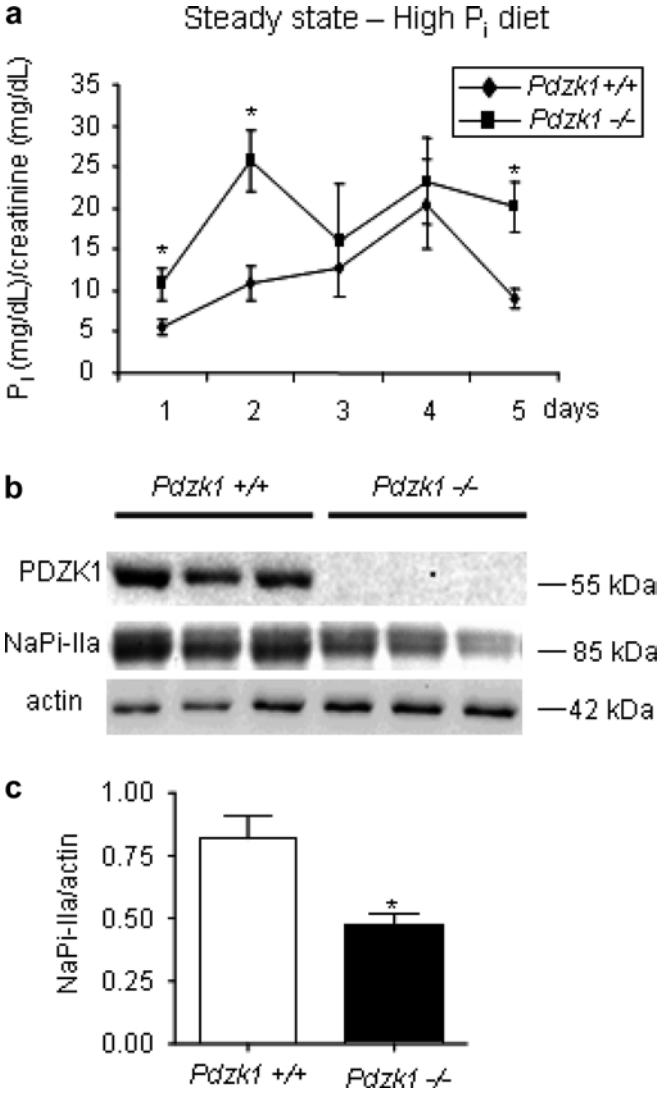

Fig. 2a–c Urinary P_i excretion and NaPi-IIa abundance in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice fed a high-P_i diet (1.2% P_i content) for 5 days. **a** Urinary P_i excretion relative to creatinine excretion (mg Pi/mg creatinine) measured in wild-type and PDZK1-deficient mice by collecting spontaneous urine samples. *Points* represents the average of the values obtained from three to five animals. *$P < 0.05$. **b**, **c** Western blot analysis for NaPi-IIa in renal brush border membranes from wild-type and PDZK1-knock-out mice after 5 days on the high-P_i diet. **b** Representative Western blot. *Upper panel*: PDZK1 expression; *middle panel*: NaPi-IIa expression. All membranes were stripped and reprobed for actin (lower panel) to normalize for loading. **c** Abundance of NaPi-IIa relative to that of actin. *$P < 0.05$

Table 1 P_i concentrations in serum and urine from wild-type (*Pdzk1*$^{+/+}$) and PDZK1-deficient (*Pdzk1*$^{-/-}$) mice under various dietary conditions. All values were obtained from animals after 5 days on either a low- or a high-$P_i$diet. Means ± SEM, $n = 6$ per group (FE_{Pi} fractional excretion of P_i)

	Pdzk1$^{+/+}$	*Pdzk1*$^{-/-}$	Significance
Serum (mg/dl)			
Low P_i	13.4 ± 0.2	14.0 ± 0.4	n.s.
High P_i	26.9 ± 3.0	24.6 ± 2.1	n.s.
Urine (mg/mg creatinine)			
Low P_i	0.08 ± 0.01	0.07 ± 0.01	n.s.
High P_i	9.00 ± 1.10	20.15 ± 3.10	$P = 0.019$
FE_{Pi} % (low P_i)	0.09 ± 0.02	0.08 ± 0.02	n.s.
FE_{Pi} % (high P_i)	6.02 ± 0.78	19.12 ± 3.56	$P = 0.020$

(Fig. 2b,c). The localization of NaPi-IIa in kidneys from wild-type and PDZK1-deficient mice displayed the same subcellular pattern and distribution along the nephron segments and generation (i.e. brush border membrane localization in the early proximal tubule of juxtamedullary nephrons) (Fig. 3). Thus, loss of PDZK1 seems to reduce urinary Pi-excretion and steady-state expression levels of NaPi-IIa in the brush border membrane of animals kept on a P_i-rich diet.

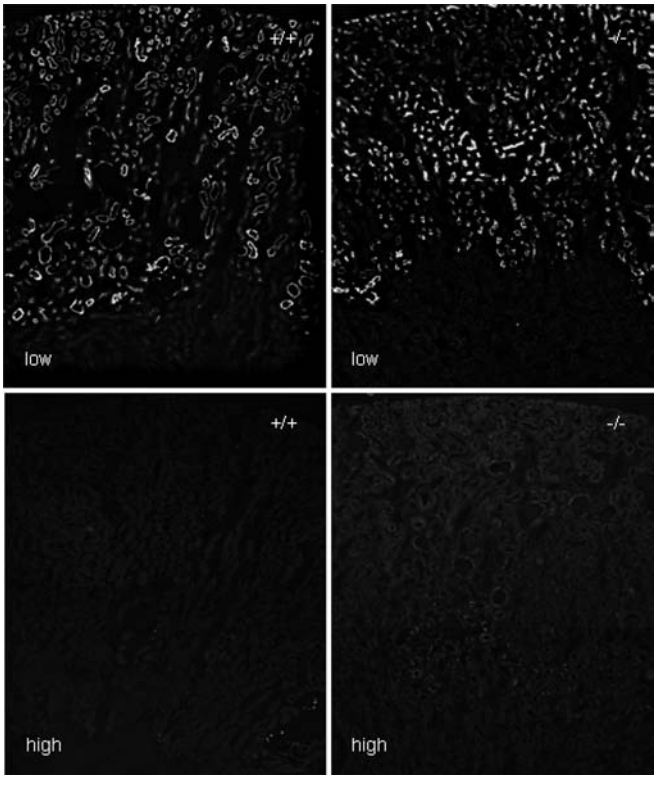

Fig. 3 Chronic adaptation to low- and high-P_i diets. Overview pictures from whole kidneys from wild-type and PDZK1-deficient mice kept for 5 days on the low- (0.1%) or high-Pi (1.2%) diet. No difference in the localization of the NaPi-IIa cotransporter could be detected. Original magnification 40×

Chronic adaptation to low- and high-P_i diet

As indicated from the steady-state results reported above, the chronic adaptation of renal P_i handling to changes in dietary P_i intake was similar in both groups of animals with the exception of lower NaPi-IIa protein levels during a P_i-rich diet. This was confirmed further by a direct comparison of the NaPi-IIa protein abundance in the brush border membrane of chronically (5 days) adapted animals. As summarized in Fig. 4, *Pdzk1* knock-out mice were as able as the wild-type animals to adapt to the different diets, with a stronger down-regulation of NaPi-IIa protein in the animals fed the high-P_i diet. Whether these lower levels of protein abundance reflect a reduction in NaPi-IIa trafficking or stability (i.e. increased turn-over in the membrane) remains unclear at this point.

Rapid adaptation to changes in dietary P_i intake

NaPi-IIa protein abundance and urinary P_i excretion adapt rapidly to acute changes in dietary P_i intake [17]. To establish whether PDZK1 plays a role in the rapid adaptive increase or decrease of NaPi-IIa abundance after ingestion of a diet either rich or low in P_i content, *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ were trained for 5 days to have access to their food for only 1 h in the morning. Some 4 h after the start of feeding, samples were collected for analysis (see Materials and methods). Animals from both genotypes were kept on low- or high-P_i diets and subdivided further into two groups, either continuing on a the prior low- or high-P_i diet or switched on day 5 to a high- or low-P_i diet respectively, thus resulting in four subgroups for each genotype.

The first group of mice, chronically adapted to the low-P_i diet, adapted rapidly to the high-P_i diet. Urinary P_i excretion was similar in both wild-type and knock-out groups on the low-P_i diet and after the switch to the high-P_i diet (Fig. 5a). These data were confirmed by Western blotting, showing similar protein abundance in the brush border membrane of both groups (data not shown). *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ control groups continued to receive a low-P_i diet and showed no difference in NaPi-IIa abundance and urinary P_i excretion as described above for steady-state conditions.

The second group of mice was switched from a chronic high- to the low-P_i diet. Again, urinary measurement of P_i excretion was comparable in wild-type and knock-out groups after the acute switch from the high- to the low-P_i diet (Fig. 5b). The higher P_i excretion under steady-state conditions in the knock-out mice group confirmed the data described above and reflected the lower protein abundance of the NaPi-IIa cotransporter in the brush border membrane as shown by Western blotting.

As shown in Fig. 6 the acute adaptation to the different diets resulted in a similar pattern of NaPi-IIa distribution in the kidney along the nephron. We found

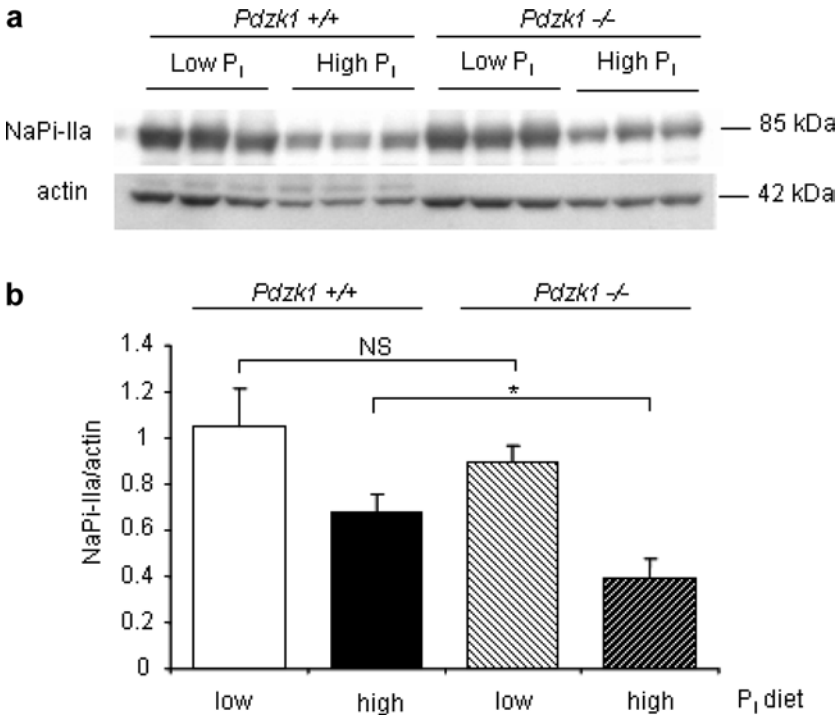

Fig. 4a,b Chronic adaptation of NaPi-IIa expression to low- and high-P_i diets. **a**, **b** Western blot analysis for NaPi-IIa in brush border membranes from kidneys of wild-type and PDZK1-knock-out mice chronically adapted to the low- and high-P_i diets. **a** Representative blot. All membranes were stripped and reprobed for actin to monitor loading. **b** Summary of NaPi-IIa abundance normalized to that of actin

no differences in the subcellular localization of the NaPi-IIa cotransporter between the wild-type and the knock-out animals in either the group of mice switched acutely from the low- to the high-P_i diet or in the group switched acutely from the high- to the low-P_i diet.

Rapid hormonal regulation of NaPi-IIa

PTH induces rapid down-regulation of NaPi-IIa by internalizing and subsequently degrading the protein [22]. PTH acts via receptors localized in the brush border membrane and on the basolateral side, activating several signalling cascades via protein kinases A and C [22]. cGMP-dependent internalization has also been described [1]. Thus, we tested whether the loss of PDZK1 affected PTH-induced internalization and degradation of NaPi-IIa in vivo and in freshly isolated kidney slices. *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice were given either 0.5 μg 1–34-PTH/g body weight i.p. or vehicle (0.9% NaCl). Mice had been adapted previously to the low-P_i diet to increase the renal expression of NaPi-IIa. 1–34-PTH reduced NaPi-IIa expression in the brush border membrane to a similar degree in both *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice (data not shown). To establish further whether the lack of the PDZK1 protein could affect the activation of these pathways or the subsequent internalization, further experiments were performed on freshly isolated kidney slices from *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice. The slices were incubated with 8-Br-cAMP (100 μM), 8-Br-cGMP (1 mM) or the protein kinase C activator DOG (10 μM) for 45 min. 1–34-PTH, which activates both cAMP/PKA and PLC/PKC dependent cascades, and fragment 3–34-PTH, which activates only the cAMP/PKA pathway, were also tested. All treatments resulted in the internalization of NaPi-IIa and a reduction of immunostaining due to the subsequent degradation. Figure 7a,c shows overviews of kidney sections from wild-type and knock-out animals with no detectable differences between the two groups. Also, as demonstrated at higher magnification (Fig. 7b,d), all treatments resulted in similar internalization of NaPi-IIa in both groups of animals.

Other proteins interacting with PDZK1

As shown previously, PDZK1 interacts via its four PDZ domains with various proteins expressed in the proximal tubule [10] such as NHE3, NHERF1, D-AKAP2, CFEX/PAT1 (SLC26A6) and the MAP17 protein. Western blot analysis detected no difference in the abundance of D-AKAP2 or MAP17 (data not shown). However, the abundance of CFEX/PAT1 (SLC26A6) was reduced in kidneys from *Pdzk1*$^{-/-}$ mice on the high-P_i diet (Fig. 8b,c). The abundance of NHERF1 in the brush border membrane of *Pdzk1*$^{-/-}$ was also increased, but only in animals on the high-P_i diet (Fig. 8b,c). The localization of all proteins investigated was undistinguishable in kidneys from wild-type and PDZK1-deficient mice (Fig. 8a).

Discussion

Renal P_i reabsorption depends critically on the expression and function of the Na^+/phosphate cotransporter NaPi-IIa as evident from studies in knock-out mice [20].

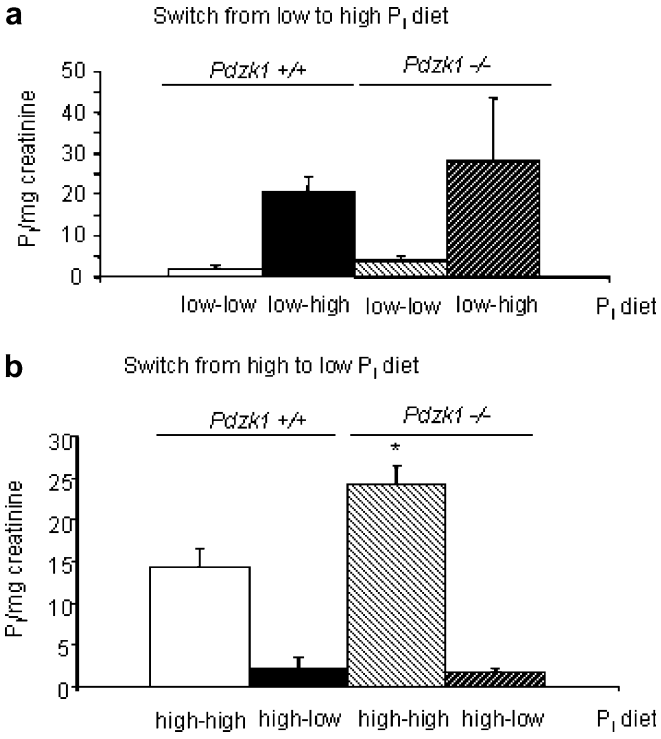

Fig. 5a,b Rapid adaptation of the NaPi-IIa cotransporter to acute changes in dietary P_i intake. Mice of both genotypes received a low-P_i (0.1%) diet (**a**) or a high-P_i (1.2%) diet (**b**) for 5 days. On day 5 some animals were then switched acutely to the other diet (high- or low-P_i diet, respectively), the remainder continued on the original diet. Urinary Pi excretion relative to creatinine excretion (mg Pi/mg creatinine) was measured in 12 wild-type and 12 PDZK1-deficient mice by collecting spontaneous urine samples. $*P < 0.05$ vs. other genotype

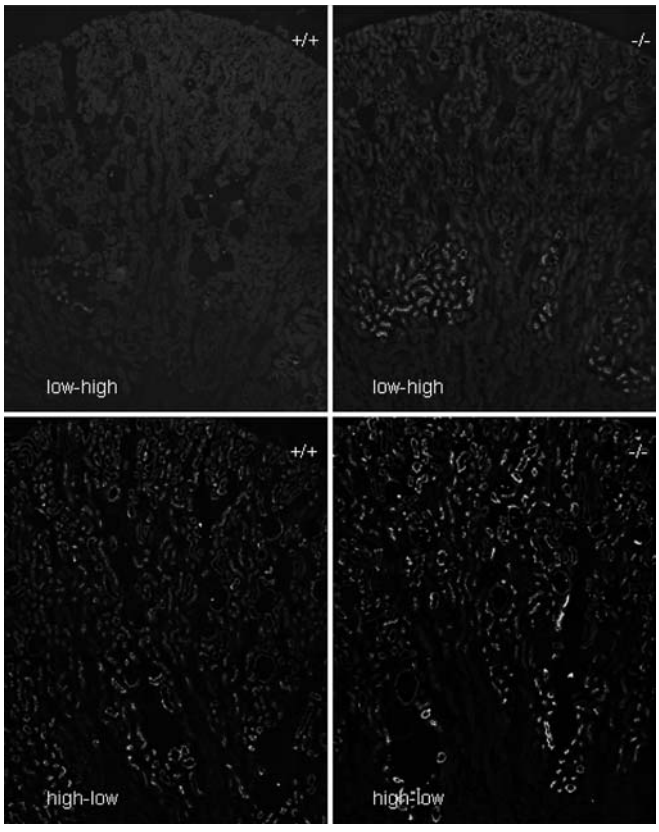

Fig. 6 NaPi-IIa specific immunoreactivity in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice kept for 5 days on either a low- (0.1%) or high-Pi (1.2%) diet and then switched acutely to the high- or low-P_i diet, respectively. Kidneys were harvested after 4 h and slices stained for NaPi-IIa-specific immunoreactivity

The activity of this transporter is regulated by a variety of hormones as well as dietary P_i intake [22]. An increase of activity is paralleled by new synthesis of transporter proteins and exocytotic insertion into the brush border membrane. In contrast, all stimuli identified so far leading to down-regulation induce retrieval of the transporter from the apical membrane, routing to lysosomes and subsequent degradation. Trafficking of newly synthesized protein to the apical membrane, anchoring in the brush border membrane and internalization and transport to the lysosomes require complex interactions with specialized proteins involved in the respective tasks. In an approach aimed at identifying such proteins important for the regulation of NaPi-IIa, several PDZ-domain-containing proteins have been found and shown to interact in vitro with NaPi-IIa through a classic PDZ-binding motif in the latter's C-terminus [8]. Among these proteins, NHERF1 and PDZK1 are of particular interest, as both proteins have been shown to interact with a variety of membrane proteins such as transporters or receptors and also with signalling proteins like protein kinase A-anchoring proteins or phospholipase C [10, 12, 19]. In addition, both proteins can also interact with each other [10, 26]. This has led to a concept suggesting that NHERF1 and PDZK1 may be part of an apical scaffolding protein network that may be important for positioning of several proteins in close proximity to each other. Such scaffolding may be important for processes like signalling or insertion into and retrieval from the apical membrane [9, 10].

The important role of NHERF1 in NaPi-IIa function and regulation has been highlighted by findings in OK cell cultures and in a NHERF1-deficient mouse model. However, little is known about the function of PDZK1 since no appropriate cell culture model has been available. The recent generation of a PDZK1-deficient mouse has now made it possible to investigate the role of PDZK1 in NaPi-IIa expression and regulation. The *Pdzk1*$^{-/-}$ mouse does not display an overt renal phenotype but does show a defect in liver lipoprotein metabolism due to a reduced expression of the high-density lipoprotein receptor scavenger receptor class B type I [15, 16].

In the present study we addressed four aspects of renal P_i handling in *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice. (1) Urinary P_i excretion, expression and localization of NaPi-IIa under steady-state conditions. These experiments showed increased P_i loss in the urine under a high-P_i diet in PDZK1-deficient mice. (2). Acute and chronic adaptation of NaPi-IIa to changes in dietary P_i intake. Here we found

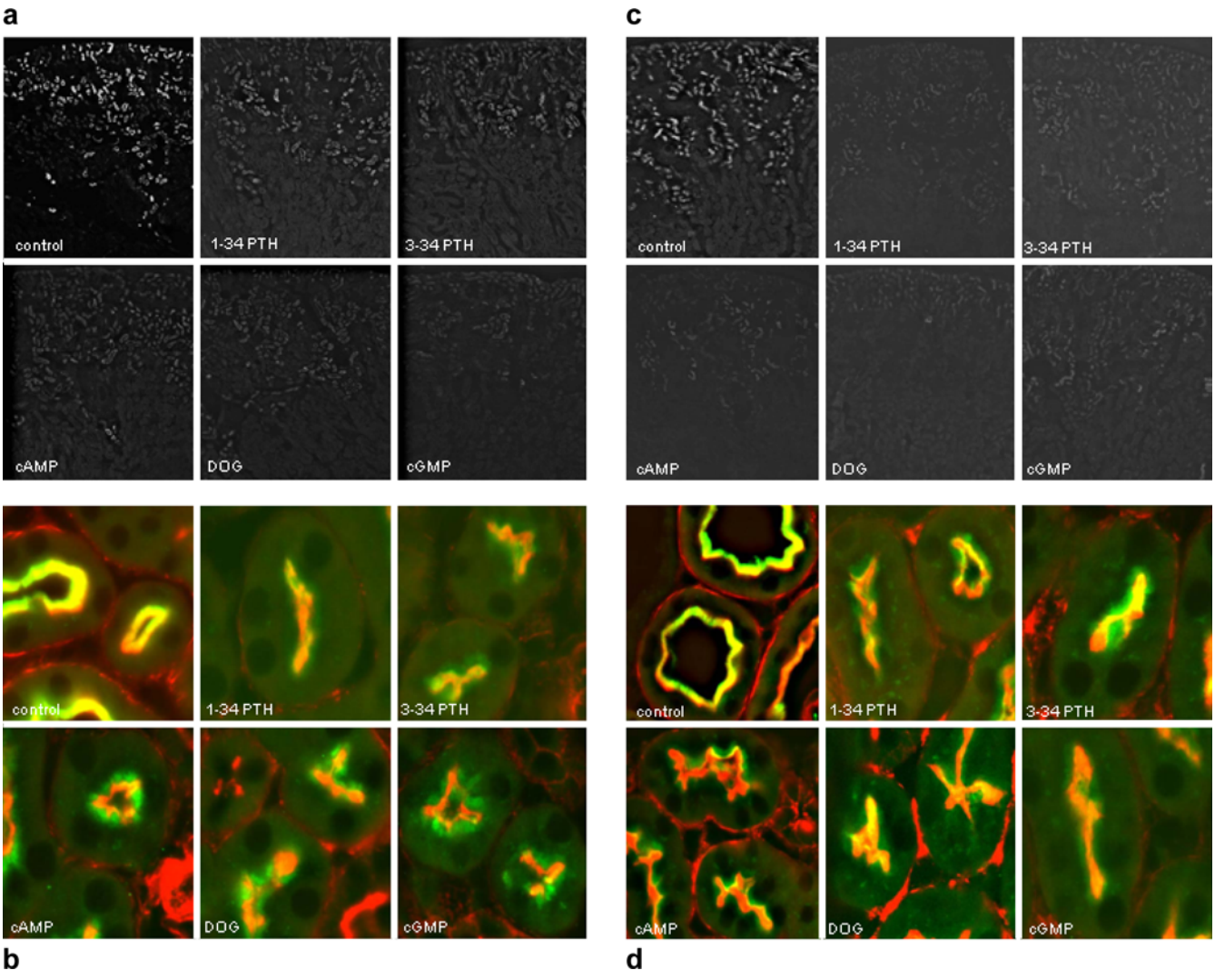

Fig. 7a–d Normal internalisation of NaPi-IIa in response to activation of various down-regulatory signalling cascades in freshly isolated kidney slices. Kidney slices from wild-type (**a**, **b**) and PDZK1-deficient mice (**c**, **d**) were prepared and incubated for 45 min either with control solution, parathyroid hormone (PTH) fragment 1–34, (*1–34-PTH*, 100 nM), 3–34-PTH (100 nM), 8-Br-cAMP (100 μM), the protein kinase C activator 1,2-dioctanoyl-sn-glycerol (*DOG*, 10 μM), or 8-Br-cGMP (1 mM). The sections were stained with an antibody against NaPi-IIa (*green*) and with rhodamine-phalloidin against *β*-actin filaments (*red*) as a marker for the brush border membrane. The high degree of overlap (*yellow*) between NaPi-IIa (green) and actin (red) under control conditions indicates localization of NaPi-IIa in the brush border membrane in both genotypes. After exposure to PTH fragments 1–34 and 3–34, cAMP, DOG or cGMP, NaPi-IIa cotransporter-related fluorescence in the wild-type brush border decreased, whereas the subapical cytoplasm showed a weak but distinct signal. There was no detectable difference between the wild-type and the knock-out animals. **a**, **c**: original magnification 40×, **b**, **d**: original magnification 800×

no difference between control and knock-out animals. (3). Acute down-regulation by internalization after activation of PTH receptors or separate activation of down-regulatory signalling cascades, again without any evident difference between control and knock-out mice. (4). The expression and localization of other PDZK1 interacting proteins than NaPi-IIa. These experiments showed the expression of the anion exchanger CFEX/PAT1 (SLC26A6) to be reduced and NHERF1/2 abundance increased under high-P_i diet conditions.

Taken together, these findings suggest that PDZK1 plays no role in the acute regulation of NaPi-IIa either by hormones or dietary changes in P_i intake. However, PDZK1 might be important for trafficking or stability of NaPi-IIa under conditions of a chronically high P_i intake. Interestingly, it has been shown that loss of NHERF1 reduces the adaptive insertion of NaPi-IIa into the membrane in response to a reduced dietary P_i intake [5, 28]. Thus, NHERF1 may be important for the trafficking or insertion of NaPi-IIa into the membrane whereas PDZK1 might serve a stabilizing function, which is unmasked only under conditions under which NaPi-IIa expression is low and not compensated by a high synthesis and insertion rate. The reduced abundance of the anion exchanger CFEX/PAT1 was only seen with the high-P_i diet. At the moment the physio-

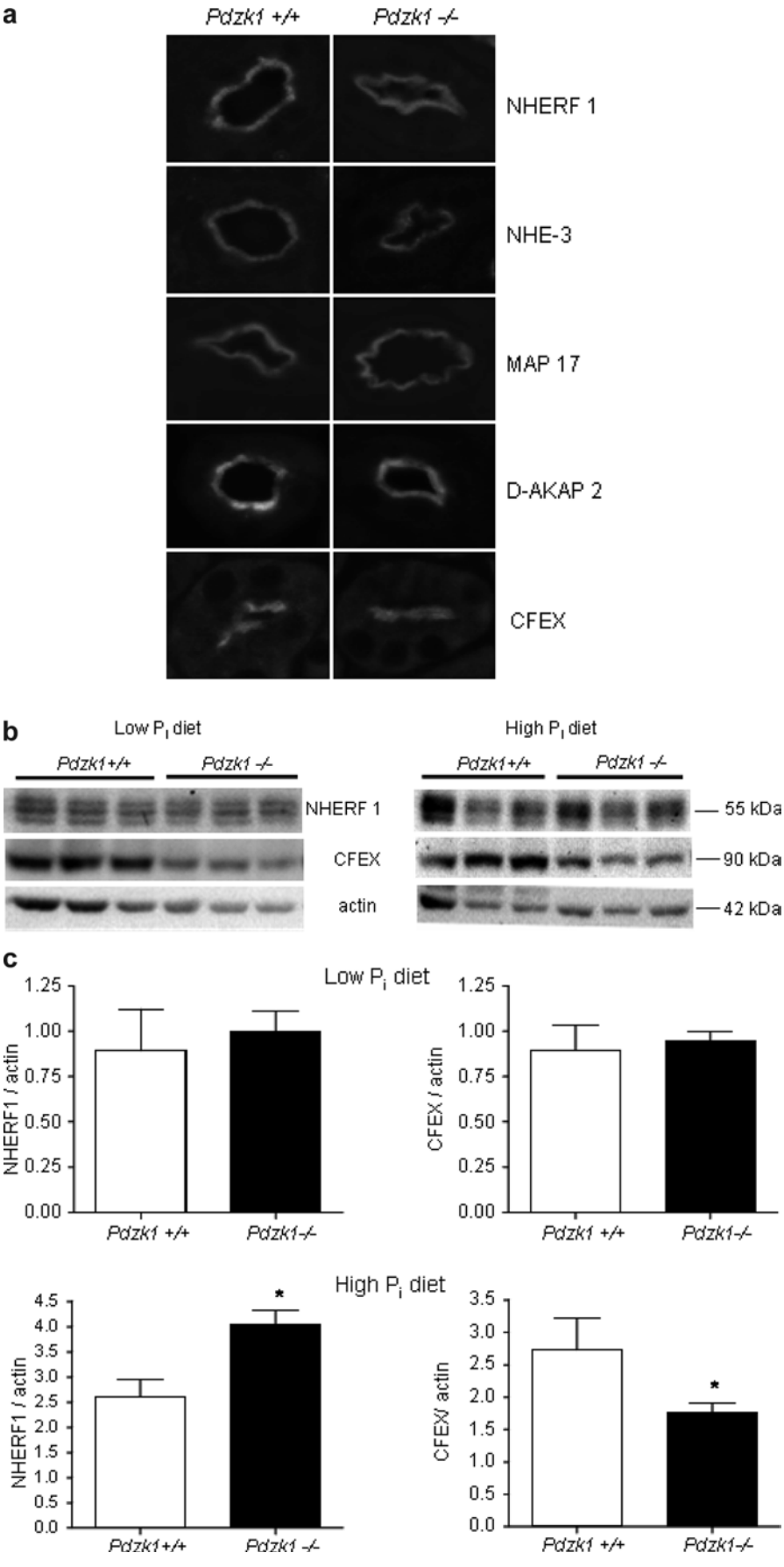

Fig. 8a–c Localization and abundance of other PDZK1-interacting proteins expressed in the proximal tubule. The localization and abundance of several proteins interacting in vitro with PDZK1 was examined in kidney sections (**a**) and in brush border membrane preparations (**b**). **a** Immunohistochemical staining for some of the NaPi-IIa interacting proteins. The localization of Na^+/H^+ exchanger-3 (*NHE3*), the NHE regulating factor-1 (*NHERF1*), the protein kinase A anchoring protein (*D-AKAP2*), the membrane-associated protein of 17 kDa (*MAP17*) and the chloride-formate exchanger (*CFEX*) was not altered in the knock-out mice, compared with wild-type. **b** Western blots of brush border membranes showing that the high-P_i diet enhanced the abundance of NHERF1 in the knock-out animals compared with wild-type and the decreased expression of CFEX. **c** Summary of NHERF1 and CFEX abundance normalized to that of actin in the brush border membrane of wild-type and PDZK1-deficient animals under low- and high-P_i diets. $^*P < 0.05$

logical significance of this finding is unclear as the physiological role of CFEX/PAT1 has not been clarified fully. The fact that NHERF1 abundance is increased under a high-P_i diet where NaPi-IIa abundance was also decreased suggests that NHERF1 up-regulation might be compensatory but not completely sufficient to prevent the reduction of NaPi-IIa expression.

Acute and steady-state levels of NaPi-IIa protein abundance are regulated by a variety of hormones including, most prominently, PTH and vitamin D_3. Even though we did not measure the concentrations of these hormones it appears very unlikely that loss of PDZK1 altered the levels of these hormones as detailed blood chemistry did not reveal any differences for other electrolytes influenced by these hormones e.g. Ca^{2+}, between *Pdzk1*$^{+/+}$ and *Pdzk1*$^{-/-}$ mice [15]. Thus, the observed changes in NaPi-IIa expression are most likely due to direct effects of PDZK1 deficiency in the proximal tubule.

In summary, loss of PDZK1 in the proximal tubule affects the expression of the major Na^+/phosphate cotransporter NaPi-IIa under conditions of a high P_i intake reducing its expression in the brush border membrane and increasing urinary P_i excretion. The acute and chronic adaptive and rapid hormonal regulation of NaPi-IIa was not altered in PDZK1-deficient mice. The subtle change in NaPi-IIa expression found in PDZK1-deficient mice may be due to compensatory or redundant processes as suggested by the fact that other PDZ proteins such as NHERF1 share many interacting partners with PDZK1. Combined ablation of both NHERF1 and PDZK1 may shed some light on the significance of their overlapping specificities.

Acknowledgements We thank E. Weinman, University of Maryland, P.S. Aronson, Yale University, and O.W. Moe, University of Texas, Dallas for providing us with antibodies against NHERF, CFEX, and NHE3, respectively. This study was supported by a grant from the Swiss National Research foundation to H.M (31-65397.01) and by grants from the Beth Israel Deaconess Medical Center Pathology Foundation, Inc., the Nell and Nancy Fund, a Cancer Prevention Fund supported by AFLAC Incorporated and The Pine Mountain Benevolent Association to O.K.

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